Amylin deposition in the kidney of patients with diabetic nephropathy

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Amylin (islet amyloid peptide) plays a critical role in islet amyloidosis and in the development of β-cell dysfunction in patients with diabetes; however, the involvement of amylin in renal amyloidosis has not been studied. For this reason, we surveyed 149 patients with biopsy-proven diabetic nephropathy (DN). The results were compared to 95 renal disease control patients, which included membranoproliferative glomerulonephritis, light-chain deposition, IgA nephropathy, and obesity-related glomerulopathy (ORG). Seventy-two of the 149 patients with DN showed amylin deposition in their renal tissue. Amylin was mainly distributed in the expanded mesangial area, Kimmelstiel–Wilson nodules, Bowman's capsule, and in blood vessels. The frequencies of mesangial proliferation, glomerular nodule lesions, and glomerular sclerosis were higher in DN patients with amylin deposits. Furthermore, the tubular interstitial lesions were more severe in these patients. Of the 95 disease-control patients, four with ORG were positive for renal amylin deposits. Our study has found renal amylin deposition in patients with DN and that the deposition was associated with disease severity. We suggest that strict metabolic control and reversing insulin resistance in patients with diabetes may blunt the process of amylin deposition in the kidney and possibly protect renal function in these patients.

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Insulin resistance and islet β-cell dysfunction are two key events in the development of type II diabetes mellitus (DM). Islet amyloidosis is a major cause of islet β-cell dysfunction and was found in 70–90% of patients with type II DM at autopsy.[1–3] It was found that the amyloid fibrils, deposited in the pancreatic islets are composed of islet amyloid polypeptide, also known as amylin.[4–6] As amylin and insulin share common promoter elements, hyperinsulinemia inevitably accompanies by hyperamylinemia. Actually, plasma amylin level is elevated in the type II diabetic patients[7] and immunoreactive amylin is present in the urine of these patients.[8]

The cytotoxic effects of amylin to β-cell within the islet were well documented. The islet amylin fibril deposition is associated with 40–50% decreased β-cell mass and β-cell dysfunction.[2,3,9–11] In addition to the direct effects of amylin on islet β-cell,[12] amylin aggregation may also participate in stimulating lipolysis, elevating plasma free fatty acid level,[7] activating the renin angiotensin aldosterone system,[13] stimulating advanced glycosylation endproducts (AGEs) receptors, and promoting inflammatory process[7,13] in patients with diabetes.

The characteristic pathological changes of diabetic nephropathy (DN), such as glomerular mesangial matrix expansion, glomerular basement membrane thickness and the Kimmelstiel–Wilson (K-W) nodules formation, indicate abnormal accumulation of extracellular matrix. Some pathogenic molecules may contribute to the formation of these lesions. As a kind of amyloid protein, amylin can form β-sheet structure and deposit in the tissue. We know that kidney is the most common involved organ in systemic amyloidosis. It is rational to assume that amylin may deposit in the renal tissue in patients with hyperamylinemia and abnormal glucose metabolism. Here, we first describe the renal amylin deposition and its role in renal tissue damage in patients with DN.

RESULTS

Amylin deposition in the renal tissue of patients with DN

Amylin deposition was found in 72 of 149 patients (48.3%) with DN. Amylin mainly distributed in the expanded mesangial area, K-W nodules, the Bowman's capsule, interstitium as well as the blood vessels (Figure 1a–d). The amylin staining in normal pancreatic tissues of patients

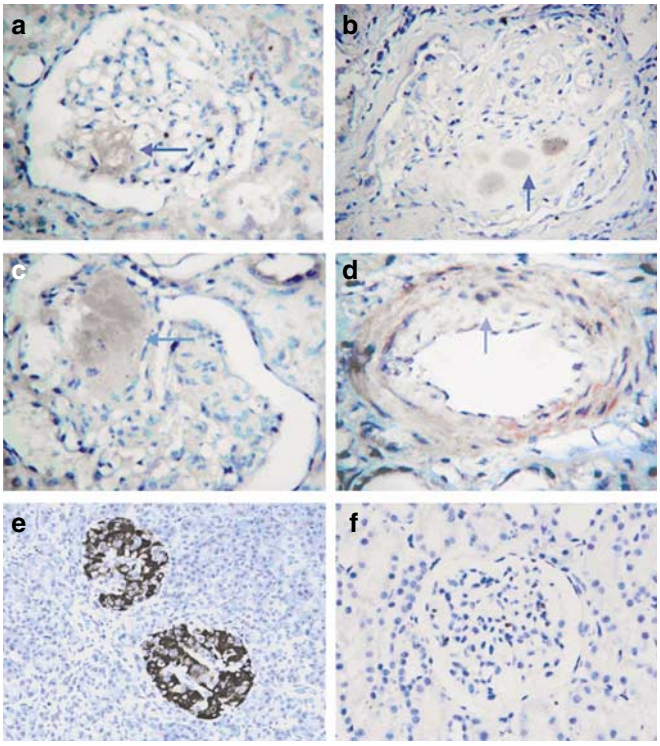

Figure 1 | Amylin deposition in the renal tissue of patients with DN. Amylin deposit was observed in the (**a**) expanded mesangial area, (**b**) glomerular segmental sclerosis, (**c**) K-W nodular lesion, and (**d**) the blood vessel. The renal amylin staining showed (**e**) positive in normal pancreatic tissues and (**f**) negative in normal control (original magnification $\times 400$).

showed positive (Figure 1e). The renal amylin staining in five normal controls showed negative (Figure 1f).

To further confirm the specialty of renal amylin deposition in patients with DN, several kinds of controls were designed for this purpose. Glomerular nodular lesion: patients with membranoproliferative glomerulonephritis (MPGN, $n=18$) and patients with light-chain deposition disease (LCDD, $n=15$); glomerular mesangial proliferative lesion: patients with IgA nephropathy (IgAN, $n=30$); Patients with metabolic disorder: obesity-related glomerulopathy (ORG, $n=32$). There was no amylin deposition in the renal tissue of patients with MPGN, LCDD, and IgAN. Interestingly, four (12.5%) out of 32 ORG patients showed amylin deposition in the renal tissue. Amylin distributed in glomerular mesangial area, Bowman's capsule, and blood vessels (Table 1).

Comparison of renal pathological changes in DN patients with or without amylin deposition

It was found that the frequency of diffuse mesangial proliferation was higher in patients with amylin deposition as compared to patients without amylin deposition (75 vs 48.6%, $P<0.005$). Both the glomerular nodular lesions and glomerular sclerosis proportion ($>15\%$) were also higher in patients with amylin deposition than those without amylin deposition. And also the tubular interstitial lesions were more severe in patients with amylin deposition (Table 2).

Table 1 | Renal amylin deposition in patients with DN and other relevant diseases

	DN	MPGN	LCDD	IgAN	ORG
n	149	18	15	30	32
Amylin deposition	72 (48.3%)	0	0	0	4 (12.5%)
Glomeruli	P	N	N	N	P
Blood vessels	P	N	N	N	P
Interstitium	P	N	N	N	P

DN, diabetic nephropathy; IgAN, IgA nephropathy; LCDD, light-chain deposition disease; MPGN, membranoproliferative glomerulonephritis; N, negative; ORG, obesity-related glomerulopathy; P, positive.

Table 2 | Comparison of renal pathological changes in patients with or without amylin deposition

	Amylin deposition		
	Positive (%)	Negative (%)	*P*-value
n	72	77	
Glomerular lesions			
Diffuse mesangial proliferation	54 (75.0)	35 (48.6)	<0.005
Nodular lesions	52 (72.2)	32 (41.6)	<0.005
Glomerulose lesions ($>15\%$)	49 (68.1)	33 (45.8)	<0.005
Tubular-interstitial lesions			
Mild	7 (9.7)	34 (44.2)	<0.005
Moderate	35 (48.6)	33 (42.9)	>0.05
Severe	30 (41.7)	10 (13.0)	<0.005

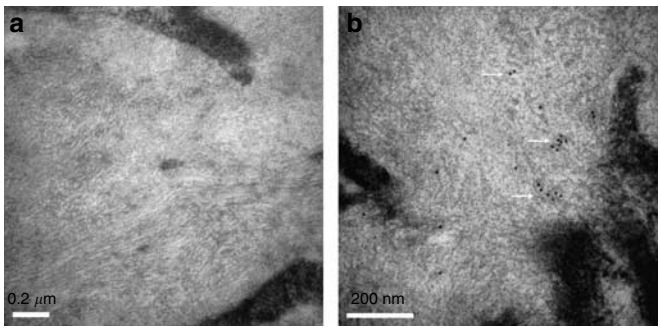

Figure 2 | Ultrastructure characteristic of amylin in the renal tissue of patients with DN. (**a**) The fibril deposits were found in the mesangium of DN patients with glomerular positive amylin staining. (**b**) Immunogold EM with an antibody to the amylin revealed the gold particles in the fibrils.

Ultrastructure characteristic of amylin in the renal tissue

The fibril deposits were found in the mesangium of DN patients with glomerular positive amylin staining. The appearance of fibrils showed to be straight, long, and non-branching with a diameter of 12–16 nm. In most cases, the fibrils were randomly arranged in the mesangium and in the glomerular basement membrane. But some deposits were seen in a tightly packed, parallel arrangement (Figure 2a). Immunogold electron microscopy (EM) with an antibody to the amylin revealed the gold particles in the fibrils (Figure 2b).

Comparison of plasma amylin level in DN patients with or without amylin deposition

The fasting plasma amylin level was detected in patients with amylin deposition ($n=18$), patients without amylin deposition ($n=34$), and the age/sex matching normal controls ($n=17$). The results were 31.0 ± 15.2, 30.5 ± 10.1, and 21.3 ± 4.6 pmol/l, respectively. The plasma amylin level was significantly higher in patients with DN than normal controls ($P<0.01$). But there were no statistical difference between DN patients with or without amylin deposition. The fasting plasma amylin level of 11 ORG patients were 26.13 ± 4.86 pmol/l. It was higher than normal controls ($P<0.05$) but lower than DN patients ($P<0.05$).

Comparison of clinical manifestations in DN patients with or without amylin deposition

There were no significant differences in the levels of fasting glucose, postprandial glucose, glycated hemoglobin, triglycerides, and cholesterol between patients with or without amylin deposition. The fasting and postprandial insulin levels were higher in patients with amylin deposition than those without amylin deposition, but did not achieve statistical difference. The body mass index was lower in patients with amylin deposition ($P<0.05$) (Table 3).

The proteinuria and serum creatinine levels were significantly higher in patients with amylin deposition ($P<0.01$), and their serum albumin level, glomerular filtration rate, and hemoglobin level were much lower than patients without amylin deposition ($P<0.01$). Much more severe renal tubular interstitial lesions were observed in patients with amylin deposition, and they have higher urine *N*-acetyl-beta-glucosaminidase ($P<0.01$) and lower urine osmolarity ($P<0.01$) than patients without amylin deposition (Table 3).

DISCUSSION

Amylin, a kind of amyloid protein, may form β-sheet structure and deposit in the tissues.[14] It has been well documented that islet amyloid has been demonstrated in up to 90% of individuals with type II DM and the degree of pancreatic amyloidosis paralleled with the severity of the disease.[4] Islet deposition of amylin may induce β-cell damage and apoptosis to result in β-cell dysfunction and absolute loss of β-cell mass by direct oxidation, special calcium channel formation on cell membrane,[15] and increased expression of p53 and p21 in the β-cell.[16] Insulin resistance and reduced β-cell mass induced by amylin result in hyperglycemia and will further stimulate compensatory secretion of insulin and amylin in remanent β-cells. Then hyperamylinemia turned back aggravating insulin resistance and β-cell dysfunction. This vicious circle leads to β-cell failure and accelerate the progression of diabetes. Transgenic mice expressing human amylin spontaneously exhibit a diabetic phenotype, such as obesity, hyperglycemia, and hyperinsulinemia.[17] This

Table 3 | Comparison of the clinical manifestations in patients with or without amylin deposition

	Amylin deposition		
	Positive	Negative	*P*-value
n	72	77	
Gender (male/female)	48/24	48/29	>0.05
Age (years)	51.36±9.45	52.36±10.54	0.543
Initial age (years)	41.90±9.80	44.71±9.55	0.078
Duration of DM (months)	108.74±75.62	90.12±70.27	0.121
Duration of DN (months)	23.24±26.87	17.73±21.84	0.171
BMI	**24.59±4.00**	**25.95±3.57**	**0.031**
Fasting blood glucose (mmol/l)	6.79±2.69	7.11±2.38	0.439
Postprandial blood insulin (mmol/l)	12.92±5.06	11.96±4.22	0.241
Fasting blood insulin (mIU/l)	24.01±25.85	19.47±15.89	0.244
Postprandial blood insulin (mIU/l)	55.44±39.19	46.50±27.27	0.321
Glycated hemoglobin (%)	6.66±1.33	6.98±1.38	0.177
Serum albumin (g/l)	**33.95±7.91**	**37.79±7.15**	**0.002**
Serum globulin (g/l)	24.17±4.24	24.79±3.70	0.348
Serum cholesterol (mmol/l)	5.73±1.70	5.69±2.51	0.906
Serum triglyceride (mmol/l)	2.26±1.66	2.49±3.37	0.600
Serum HDL-c (mmol/l)	1.36±0.49	1.25±0.36	0.149
Serum LDL-c (mmol/l)	3.19±1.28	3.44±1.39	0.318
Hypertension *N* (%)	**70 (97.2%)**	**63 (81.8%)**	**<0.005**
Blood urea nitrogen (mg/dl)	**23.23±16.17**	**16.24±10.03**	**0.002**
Serum creatinine (mg/dl)	**1.99±1.63**	**1.16±0.80**	**<0.001**
GFR (ml/min)	**52.99±30.44**	**74.24±30.13**	**0.001**
Proteinuria (g/24 h)	**4.02±2.55**	**2.62±3.35**	**0.005**
Urine NAG enzyme (μ/g Cr)	**40.72±34.58**	**30.08±26.31**	**0.035**
Urine retinol binding protein (mg/l)	6.11±8.37	3.37±8.35	0.066
Urinary osmotic pressure (mosM/kg H_2O)	**442.93±140.30**	**594.75±211.94**	**<0.001**
Hemoglobin (g/dl)	**10.79±2.56**	**12.59±2.27**	**<0.001**

BMI, body mass index; DM, diabetes mellitus; DN, diabetic nephropathy; HDL-c, high-density lipoprotein-c; LDL-c, low-density lipoprotein-c; NAG, N-acetyl-beta-glucosaminidase. We use bold characters to outline the items which have statistical significance.

indicates that exceeding physiological concentration of amylin participate in the formation of diabetes.

As amylin plays so important role in the insulin resistance and diabetic-associated tissue injury, the role of amylin in diabetic renal lesions should not be ignored. Our results first indicated that 48.3% of patients with DN have renal amylin deposition. The specialty meaning of amylin deposition found in DN patients was confirmed by the disease controls. Amylin was not found in the renal tissue of patients with MPGN, LCDD, and IgAN. The renal amylin deposition mainly distributed in expanded mesangial area, K-W nodules, thickened Bowman's capsule, and injured blood vessel wall in patients with DN. And this kind of distribution was corresponding to characteristic renal lesions in DN. For instance, the frequency of glomerular nodular lesions, glomerulosclerosis, and the degree of tubular interstitial lesions are more severe in patients with amylin deposition than patients without renal amylin deposition. Consistent to these, the 24-h urine protein excretion, urinary *N*-acetyl-beta-glucosaminidase level, blood pressure, and serum creatinine level were elevated significantly in patients with renal amylin deposition. Furthermore, the glomerular filtration rate as well as hemoglobin level was markedly decreased in patients with amylin deposition. These findings indicate that renal amylin deposition may participate the process of diabetic renal lesion.

Exogenously added human amylin is cytotoxic and induces apoptosis when added to a variety of cultured cells, including primary rat pancreatic cells, hippocampal neurons, aortic endothelial cells, PC12 pheochromocytoma cells,[18] and COS cells.[19] To explore the underlying mechanisms of amylin deposition-induced renal tissue injury in patients with DN, other previous studies demonstrated that amylin is able to induce mesangial cell apoptosis and increase the permeability of endothelial cells *in vitro*.[20] These effects may contribute to the renal damage mediated by amylin deposition in patients with DN. Also Cooper and co-workers had demonstrated that amylin intermediates rather than amylin-associated amyloid are more responsible for β-cell toxicity.[21] So amylin oligomers may be more toxic than amyloid itself in diabetic renal lesion. In addition, amylin may deposit in renal tissue by binding with its corresponding receptor. The known amylin receptor-like molecules include receptor for advance glycosylation endproducts (RAGEs)[22] and calcitonin receptor.[23] As RAGE was expressed in the glomerular visceral epithelial cells (podocytes),[24] it is assumed that amylin may bind with RAGE on podocytes. RAGE was activated when binding with its ligand – amylin. Then several signal-transduction pathways involved in inflammation were activated, such as P21 Ras, mitogen-activated protein kinase, nuclear factor-κB, and CDC42/rac pathway.[25] Activation of RAGE may induce vascular endothelial growth factor expression, which may induce vascular endothelial cell hyperpermeability, upregulate intracellular adhesion molecule-1 expression, promote adhesion and inflammation of macrophage cells, activate nuclear factor-κB, and increase the secretion of proinflammatory factors such as tumor necrosis factor-α, interleukin-1β, and interleukin-6[26] and involved in the podocyte injury and the development of proteinuria. Of course, this needs to be clarified. It has been reported that renal amylin deposition can trigger the activation of renin angiotensin aldosterone system. Wookey *et al.*[27] demonstrated that there was a significant correlation between the increased density of amylin binding sites in the renal cortex and the rise of systolic blood pressure in two animal models of hypertension. The higher frequency of hypertension on patients with amylin deposition found in this study supports the correlation between renal amylin deposition and increased blood pressure.

It has been revealed that the increased secretion of amylin and elevated circulating amylin concentration will promote the formation of pancreatic islet amylin deposition.[28,29] To answer why only a part of DN patients showed amylin deposition in the renal tissue, the plasma amylin concentration was measured in these patients. The plasma amylin concentration was significantly higher in patients with DN than normal controls. However, no difference was found between DN patients with or without renal amylin deposition. This result indicated that increased plasma amylin concentration is necessary but not the only factor that determines the renal amylin deposition. In the state of DM, hyperglycemia and hyperlipidemia-induced glycotoxicity, lipotoxicity, and redox stress may promote and accelerate islet amyloid formation. AGEs may modify amylin protein structure squinting toward crosslink and assembly in post-transcription mode and accelerate the islet amyloid deposition.[30,31] Thus, comparison was made in the abnormalities of glucose and lipid metabolism between DN patients with or without renal amylin deposition. Although there were no difference found between them. The therapeutic intervention in these patients could not been ruled out. Note that, four out of 32 patients with ORG showed renal amylin deposition further illustrate the correlation between metabolic disorders and renal amylin deposition. Insulin resistance is a common metabolic disorder in patients with ORG,[7] the elevated plasma amylin level found in this group of ORG patients not only indicate the presence of insulin resistance in these patients, but also demonstrate the correlation between the metabolic disorder and the deposition of amylin in the renal tissue, and may extend this issue to renal protection in patients with ORG or metabolic syndrome.

We first time described that renal amylin deposition was found in patients with DN, and the deposition associated with the disease severity. In addition to the hyperamylinemia, the metabolic disorders and the change of local milieu of kidney may accelerate the formation of amylin deposition in the renal tissue. Although further studies are needed to explore the pathogenetic role of amylin in the development of diabetic renal lesions, the finding of this study indicates that strict metabolic control and reversing the state of insulin resistance in patients with diabetes may blunt the process of

amylin deposition and protect renal function in these patients.

MATERIALS AND METHODS

Patients

One hundred and forty-nine patients with biopsy-proven type II DN were selected in this study. There were 96 male and 53 female patients, age ranged from 29 to 74 years old (51.88 ± 10.01 years). All these patients met the criteria for type II DM, and the renal biopsy consistent with the diagnosis of DN and exclusion for other concomitant renal diseases. The patients' hospital records, including age, gender, course of disease, stature, avoirdupois, blood pressure, the level of fasting and postprandial blood glucose, fasting and postprandial blood insulin, glycated hemoglobin, serum albumin, globulin, cholesterol, triglyceride, high-density lipoprotein-c, low-density lipoprotein-c, blood urea nitrogen, serum creatinine, hemoglobin, glomerular filtration rate, proteinuria, urine *N*-acetyl-beta-glucosaminidase enzyme, urine retinal binding protein, and urinary osmotic pressure were obtained. The disease controls used in this study include MPGN ($n = 18$), LCDD ($n = 15$), IgAN ($n = 30$), and ORG ($n = 32$). Here, patients with ORG were defined as obesity (body mass index of 28 kg/m^2 or greater), proteinuria, and biopsy-proven ORG. Patients with MPGN, LCDD, and IgAN have normal body mass index and normal glucose metabolism. Six normal donor kidneys unfitting for transplantation served as normal controls. Five normal pancreatic tissues of patients obtained in surgery served as positive controls.

Renal histology

Renal specimens were fixed in 10% neutrally buffered formalin and embedded in paraffin using routine procedures. Sections (2 μm in thickness) were stained with hematoxylin/eosin, periodic acid-Schiff reagent, silver methenamine, and Ledewig's trichrome. Alkaline Congo Red staining was performed in five pancreatic tissues of patients with type II DM. Renal histological changes were recorded by the same pathologist in single-blind mode. Here, the mild interstitial lesions defined as accounting for less than 25% area of interstitium. The moderate interstitial lesions defined as among 26–50% area of interstitium. The severe interstitial lesions defined as more than 50% area of interstitium.[32]

Immunohistochemistry staining of amylin in renal tissue

Cryosections (4 μm) were prepared and fixed for 7 min in acetone. To observe the distribution of amylin in renal tissues, peroxidase-antiperoxidase (PAP) four-layer staining protocol was performed. Briefly, after incubation with 10% fetal calf serum for 20 min to block unspecific binding, the primary antibody (monoclonal antibody to amylin, 1:50, Serotec, Washington, DC, USA) was applied for overnight at 4°C, followed by a sequential incubation with horseradish peroxidase-conjugated secondary antibody (rabbit anti-mouse IgG, 1:100, Dako, Carpinteria, CA, USA), bridging antibody (swine anti-rabbit IgG, 1:100, Dako), and rabbit PAP (1:100, Dako) for 40 min each at room temperature. Sections were then visualized using 3,3′-diamino-benzidine and counter-stained with hematoxylin, dehydrated, and mounted with neutral resin. Negative controls were performed by replacing the primary antibody with isotype control and by disusing the treatment of the first antibody.

Measurement of fasting plasma amylin level

The plasma amylin level was measured with competitive enzyme-linked immunosorbent assay (Phoenix pharmaceutical, INS, Burlingame, CA, USA) according to the manufacturer's protocol. Five milliliters blood samples was collected from each patient and was put into the lavender vacutaner tubes that containing ethylenediaminetetraacetic acid. Then the tubes were gently rocked several times immediately after collection of blood with anti-coagulation. The blood samples were transferred from the lavender vacutaner tubes to centrifuge tubes containing aprotinin (0.6 TIU (trypsin inhibitor unit)/ml of blood, Calbiochem, San Diego, CA, USA) and gently rocked for several times. The blood samples were centrifuged at 1600 *g* for 15 min at 4°C and the plasma collected. Then the plasmas were acidified with an equal amount of buffer A (1% trifluoroacetic acid), mixed, and centrifuged at 10 000 *g* for 20 min at 4°C. A SEP-COLUMN containing 200 mg of C18 (Phoenix pharmaceutical) was equilibrated by washing the buffer B (60% acetonitrile in 1% trifluoroacetic acid) (1 ml, once) followed by buffer A (3 ml, three times). The acidified plasma solutions were loaded onto the pretreated C18 SEP-COLUMN. The column was washed slowly with buffer A (3 ml, twice) and the wash was discarded. The peptide was eluted slowly with buffer B (3 ml, once) and eluant was collected into a polystyrene tube. Eluant was evaporated to dryness and kept at −20°C. The dried extract was reconstituted by assay buffer. Fifty microliters prepared peptide standard solutions or blood samples, 25 μl rehydrated primary antiserum, and 25 μl rehydrated biotinylated peptide were added into their designated wells. And the immunoplate was incubated for 2 h at room temperature. Each well was washed with 300 μl assay buffer for five times. Hundred microliters streptavidin-horseradish peroxidase solution was added into each well and incubated for 1 h at room temperature. The immunoplate was washed and blot dried six times. Then 100 μl substrate solution provided in the kit was added into each well and incubated for 1 h at room temperature. Hundred microliters 2 N HCl was added into each well to stop the reaction. Absorbance optical density (OD) at 450 nm was read and calculated. The concentration of amylin was calculated as pmol/l.

Electron microscopy and immunogold electron microscopy

The ultrastructure was evaluated using EM. Briefly, renal tissue fragments were fixed in a cool 3.75% phosphate-buffered glutaraldehyde solution (0.2 M, pH 7.2), post-fixed in 1% osmium tetroxide, and embedded in epon. Ultrathin sections were observed and microphotographs were obtained using EM (HITACHI 7500, Tokyo, Japan).

Immunogold EM was used to find amylin deposition in renal tissue. Small renal pieces were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h and subsequently washed in PBS, then dehydrated in graded ethanols and embedded in LR White resin. Semithin sections (1 mm) were cut from epon-embedded blocks and stained with toluidine blue. Ultrathin sections (80 nm) were cut in an ultratome (Leico EM UC6), mounted on nickel grids, blocked with 2% bovine serum albumin in PBS for 10 min, and incubated with mouse anti-human amylin (Serotec, catalog number MCA1126) diluted 1:20 in 1% bovine serum albumin for overnight at 4°C. The grids were washed in PBS for 10 times and then incubated with 2% bovine serum albumin for 10 min. After discarding the liquid followed by goat anti-mouse IgG coupled to 10-nm colloidal gold (catalog number 25129, Electron Microscopy Science, Fort Washington, PA, USA), control grids were incubated with PBS substitute for mouse-anti-human amylin and by disusing the treatment of mouse-anti-human amylin. Then, grids were stained with uranyl acetate (25 min) and lead citrate (12 min) and examined with EM (HITACHI 7500, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS software (version 11.0). Data were expressed as mean $\pm$ s.d. and analyzed using the one-way analysis of variance and Student's *t*-test. Statistical significance was assumed for $P<0.05$ and very high significance levels were defined as $P<0.01$.

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